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Please send to Irene Marx, Art Unit 1651; CM1, Room 10E05, phone 308-2922, Mail box in 11B01

Suemori et al., Semei Kogaku Kogyo Gijustsu Kenkyusho Kenkyu Hokoku (1995), 3(2), 33-36

3927035

Spamins et al., J. Bacteriol., (1976), 127(1), 362-6

Karoum, F., Neuropsychopharmacol. Trace Amines:Exp. Clin. Aspects, 2nd (1985) 433-450. Ed. Boulton. Publisher: Humana, Clifton, N.J.

Blakley et al., Can. J. Microbiol., 1977, 23(9), 1128-1139

Blakley et al., Can. J. Microbiol., 1972, 18(8), 1247-55

Mills et al., Insect Biochem., 1971, 1(3), 264-70.

Kindl, H., Eur. J. Biochem, 1969, 7(3), 340-7

Fernandez-Canon et al., J. biol. chem., 1995, 270(36), 21199-205

Irene Marx
Art Unit 1651
CMI 10-E-05,
Mail Box 11-B-01
703-308-2922

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Biosynthesis and Metabolism of Hydroxyphenylacetic Acids in Higher Plants

H. KINDL

Lehrkanzel für Biochemie der Universität Wien

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1. 2-Hydroxyphenylacetic acid, a natural phenolic product found in the genus *Astilbe*, derives from the shikimic acid pathway *via* phenylpyruvic acid. The existence of two routes for the biosynthesis of 2-hydroxyphenylacetic acid could be demonstrated:

a) A direct transformation of phenylpyruvic acid into 2-hydroxyphenylacetic acid involving a migration of the side chain. More than 95% of the tritium activity of 2-hydroxyphenylacetic acid was localized in position 5 when L-[4-^{3}H]phenylalanine was fed. This complex oxidation is analogous to the known conversion of 4-hydroxyphenylpyruvic acid to homogentisic acid.

b) A hydroxylation of [4-^{3}H]phenylacetic acid to [4-^{3}H]2-hydroxyphenylacetic acid was observed *in vivo*, and was also found to take place *in vitro* utilizing the system peroxidase-endiol-O_2.

2. 2,3-Dihydroxyphenylacetic acid and 2-hydroxy-3-methoxyphenylacetic acid could be established as natural products occurring in higher plants. Their chemical synthesis is described. By feeding experiments the following metabolic pathway is suggested: 2-hydroxyphenylacetic acid → 2,3-dihydroxyphenylacetic acid → 2-hydroxy-3-methoxyphenylacetic acid. 3,4-Dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid have been detected in extracts from various species of *Astilbe* and identified by paper chromatography. These two acids are metabolic products of 4-hydroxyphenylacetic acid.

3. Experiments with [U-^{14}C]shikimic acid and DL-[α-^{14}C]phenylalanine seem to indicate that the regulation of the biosynthesis of 2-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid takes place on the level of prephenic acid.

4. Preliminary results were obtained consistent with the hypothesis that in *Astilbe chinensis* the 2,3-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 2,5-dihydroxyphenylacetic acid are further metabolized and can be degraded by ring cleavage.

Several years ago, 2-hydroxyphenylacetic acid was identified as an important abnormal metabolic product in the urine of patients with phenylketonuria [1]. Also culture filtrates from a submerged cultivation of *Penicillium chrysogenum* were found to contain 2-hydroxyphenylacetic acid [2]. Finally, in 1962 this compound was detected in higher plants [3], but it appeared to be restricted to the genus *Astilbe* (Saxifragaceae). In contrast, many plants contain 4-hydroxyphenylacetic acid [4]. It is not yet understood why *Astilbe* forms the *o*-isomer predominantly. A study of the regulations responsible for these hydroxylations seemed to be of interest.

The transformation of D- and L-phenylalanine [5,6] as well as of 2-hydroxyphenylalanine [7] into 2-hydroxyphenylacetic acid by patients with phenylketonuria was described earlier. Taniguchi and Armstrong [8] studied the conversion of phenylpyruvic acid to 2-hydroxyphenylacetic acid by enzyme preparations from various mammalian sources. In higher plants, however, to the knowledge of the author, no work has so far been done on the biosynthesis and metabolism of hydroxyphenylacetic acids. The following paper deals with the biosynthesis of six hydroxyphenylacetic acids and describes steps involved in the regulation of their formation.

Enzymes. 4-Hydroxyphenylpyruvic acid oxidase (EC 1.14.2.2); peroxidase (EC 1.11.1.7); L-amino acid oxidase (EC 1.4.3.2); L-phenylalanine hydroxylase (EC 1.14.3.1); L-phenylalanine ammonia lyase (EC 4.3.1.5).

Note. Numbers (1, 2 ... 6) are used for positions in the ring. Positions on the side chain of β-phenylpropionic acid derivatives are designated as α and β. Exceptions are made for trivial names, *e.g.* *p*-coumaric acid.

MATERIALS AND METHODS

L-[U-^{14}C]Phenylalanine, DL-[α-^{14}C]phenylalanine, DL-[α-^{14}C]tyrosine, L-[U-^{14}C]tyrosine and L-[4-^{3}H]-phenylalanine were purchased from Radiochemical Centre, Amersham. DL-[β-^{14}C]Phenylalanine was supplied by New England Nuclear. D-[U-^{14}C]Glucose was obtained by a biosynthetical method [9] while [β-^{14}C]cinnamic acid, [β-^{14}C]phenylpropionic acid, [*carboxyl*-^{14}C]phenylacetic acid and [1-^{14}C]acetic acid

could be synthesized according to well-described procedures [10]. An enzymatic oxidation of DL-[β-^{14}C]phenylalanine by L-amino acid oxidase from *Crotalus adamanteus* led to the formation of [β-^{14}C]-phenylpyruvic acid which was purified by paper chromatography and recrystallized with inactive material. [*carbonyl*-^{14}C]-Salicylaldehyde needed for the synthesis of [β-^{14}C]o-coumaric acid and [β-^{14}C]-melilotic acid could be obtained from 2-bromophenol and Na^{14}CN *via* [*nitril*-^{14}C]2-hydroxybenzonitril [11]. Attempts to determine the L-phenylalanine hydroxylase activity by the procedure of Nair and Vining [12] failed in the case of *A. chinensis*. L-Phenylalanine ammonia lyase was prepared from the acetone powder of *A. chinensis* according to Koukol and Conn [13]. After total ammonium sulfate fractionation the enzyme protein was purified by gel filtration on Sephadex G-50.

[U-^{14}C]Shikimic Acid

5 g of needles (about 7 cm long) of *Pinus strobus* were allowed to photoassimilate in a $^{14}CO_2$-atmosphere for 2 weeks in a chamber [14]. The plant material was extracted with ethanol. After dilution with 20 mg inactive shikimic acid in 20 ml water, concentration *in vacuo* yielded a thin syrup which was shaken with 100 ml ether, 100 ml 0.1 % $NaHCO_3$ solution and 1 g glass beads. The residue was filtered off and washed with water. After separation of the ether phase the aqueous solution was concentrated *in vacuo*. By stirring with 30 ml of Dowex 50x4 (200—400 mesh) the cations were removed. The exchange resin was washed and the solutions evaporated under reduced pressure. A first purification of the shikimic acid could be achieved by paper electrophoresis in pyridine acetate puffer, pH 5.0. Subsequently, the corresponding zone was eluted and further purification was carried out by preparative paper chromatography (Macherey & Nagel, 827) in acetone—water (9 : 1, v/v). In this system shikimic acid has a R_{Glc}-value (related to glucose) of 1.9. Finally, the shikimic acid eluted from the paper was recrystallized from ethanol and yielded 37 mg, m. p. 182—184°. The radiochemical yield varied between 20—32 μC per mC $Ba^{14}CO_3$. A sample of the acid was converted to 4-hydroxybenzoic acid and the CO_2 obtained after decarboxylation showed 1/7 of the entire activity.

2,3-Dihydroxyphenylacetic Acid

3.0 g of the lactone of 2-hydroxy-3-methoxyphenylpyruvic acid (from 2-hydroxy-3-methoxybenzalhydantoin [15]) were dissolved in 12 ml 2 N NaOH by heating. To the well chilled alkaline solution 2.5 ml H_2O_2 (30 %) were carefully added and the mixture allowed to stand over night. After dropwise acidification with concentrated HCl and intensive cooling 2.2 g 2-hydroxy-3-methoxyphenylacetic acid could be obtained. Recrystallization from water yielded 1.9 g. Sublimation at 10^{-3} Torr gave white crystals (m. p. 128°). Calc. for $C_9H_{10}O_4$: 59.4, C; 5.56, H; 17.0, OCH_3; Found: 59.6, C; 5.68, H; 17.1, OCH_3; 1.9 g of the former compound were refluxed with 30 ml HBr (49 %) during 10 hours. The volume was reduced *in vacuo* and the 2,3-dihydroxyphenylacetic acid was obtained by continuous extraction with ether. The residue remaining after evaporation was sublimed at 10^{-3} Torr and 190° and yielded 1.1 g lactone (7-Hydroxybenzo[b]furan-2-one), m. p. 191°.

Calc. for $C_8H_6O_3$: 63.9, C; 4.02, H;
Found 64.3 C; 4.14, H;

(4-^{3}H)Phenylacetic Acid

800 mg 4-aminophenylacetic acid were mixed with 2.0 ml ^{3}HHO and 1.2 ml HCl under intensive cooling. Maintaining a temperature of 0° 800 μl 60 % $NaNO_2$ solution were added dropwise. After addition of 1.5 g finely powdered NaH_2PO_2 the mixture was stirred for 10 hours. Then, the reddish coloured product was poured into 200 ml 1 N HCl and extracted with ether. The ether solution was separated, dried over sodium sulfate, and evaporated to dryness. The residue could be purified by sublimation at 10^{-1} Torr and yielded 210 mg, m. p. 76—78°. The radiochemical yield was 2 %. When HCl was administered as gas and $NaNO_2$ was dissolved in ^{3}HHO the yield could be raised to 4 %. It was proved that the labelling was localized in the para-position only. This was established by nitration of [4-^{3}H]phenylacetic acid leading to a 4-nitrophenylacetic acid the specific activity of which was less than 0.01 % of the starting material, phenylacetic acid.

Bromination of 2-Hydroxyphenylacetic Acid

400 mg 2-hydroxyphenylacetic were dissolved in 400 ml water and allowed to react with 350 μl Br_2. The mixture was stirred for 5 hours at 40°, cooled and extracted with chloroform. The solvent was removed and the residue recrystallized several times from ethanol—water. Yield 510 mg, m. p. 155°.

Calc. for $C_8H_6Br_2O_3$: 31.0, C; 1.96, H; 51.3 Br;
Found: 30.6, C; 2.10 H; 50.7, Br;

Nitration of Phenylacetic Acid

2.5 g Phenylacetic acid were dissolved in 5 ml conc. sulfuric acid. 1.8 ml HNO_3 ($d = 1.44$) were added keeping the solution at 20—30° while shaking. After 10 hours the yellow solution was poured into 100 ml ice-water mixture. The precipitate isolated was recrystallized several times from water—ethanol and later from acetonitril—benzene. Finally, the 4-nitrophenylacetic acid obtained showed a m. p. of 150—153°. Control experiments revealed that no exchange (less than 0.1 %) of hydrogen in the meta-

position to the side chain takes place when the nitration was carried out in mixtures containing $^3H^+$.

Decarboxylation of 4-Hydroxybenzoic Acid

100 mg 4-hydroxybenzoic acid and 150 mg copper powder were heated in 4.0 ml freshly distilled quinoline at 230° for a period of 60 min. The CO_2 formed was swept with a nitrogen stream into a trap containing 10 ml N/10 NaOH. After addition of NH_4Cl the trapped CO_2 was precipitated with $BaCl_2$. The content of the reaction vessel was diluted with ether, placed in a separatory funnel and extracted with 7 ml 6 N HCl. The ether solution was then extracted with 5 ml 1 N NaOH and the alkaline solution acidified with 0.5 ml concentrated HCl. By addition of bromine the tribromophenol is precipitated and sublimed at 10^{-3} Torr and 125°. Yield 71 %. The degradation of hydroxyphenylacetic acids was performed *via* hydroxybenzoic acid and phenol according to a procedure described earlier [16]. L-Tyrosine was converted to 4-hydroxyphenylpyruvic acid by L-amino acid oxidase (from *Crotalus adamanteus*), and 4-hydroxyphenylpyruvic acid oxidized to 4-hydroxyphenylacetic acid by alkaline H_2O_2.

Isolation of 2,3-Dihydroxyphenylacetic Acid

4 kg roots of *Astilbe chinensis* were extracted with methanol. The cooled and filtered solution was concentrated under reduced pressure to a thin syrup and then treated with 200 ml of hot water. The mixture was filtered through Celite and then exhaustively extracted with ether. The residual syrup obtained after evaporation was purified by fractional sublimation at 10^{-2} Torr and 190°. Further concentration of the product was achieved by preparative paper chromatography (on 4 sheets, each 44 cm broad, Macherey & Nagel, 218) using the solvent system L_2 (*cf.* paper chromatography). Finally, the corresponding zone was eluted and the material purified by fractional sublimation [4]. Yield: 38 mg, m. p. 190°. The isolated product had the same chromatographic and spectroscopic behaviour as the synthetic compound.

Administration of the Radioactive Precursors and Isolation of the Metabolites

Feeding experiments were carried out with leaf cuttings which were placed into conical beakers containing 5 ml of the solution of the appropriate radioactive compound (less than 1 mg/ml). The cuttings were allowed to metabolize for a period of 48 hours at room temperature under artificial light. Water was supplied as required for complete uptake of the compounds (*ca.* 5—10 hours) and for the remainder of the metabolic period. Then the leaves were extracted with ethanol. The cooled extracts were filtered and concentrated on a rotary evaporator *in vacuo*. The remaining syrup was shaken with ether and $NaHCO_3$ solution. The alkaline solution was acidified and thoroughly reextracted with ether. This ether fraction was separated by paper chromatography and the radioactivity counted. The appropriate zones were rechromatographed in another solvent. The aqueous solution after the last ether extraction contained the amino acids (*e. g.* tyrosine) which were separated by paper chromatography.

Paper Chromatography

Predominantly, 2 solvent systems were utilized. L_1: benzene—acetic acid—water (4:2:1, v/v/v), (upper phase); L_2: *n*-butanol—concentrated NH_4OH—ethanol—benzene (5:3:2:1, v/v/v/v).

Compound	R_x-values related to 2-hydroxyphenylacetic acid, in	
	L_1	L_2
Phenylacetic acid	2.28	1.12
α-Hydroxyphenylacetic acid	1.00	0.70
2-Hydroxyphenylacetic acid	1.00	1.00
4-Hydroxyphenylacetic acid	0.73	0.55
2,3-Dihydroxyphenylacetic acid	0.44	0.42
2,5-Dihydroxyphenylacetic acid	0.10	—
3,4-Dihydroxyphenylacetic acid	0.22	—
2-Hydroxy-3-methoxyphenylacetic acid	1.40	0.90
3-Hydroxy-4-methoxyphenylacetic acid	1.24	0.48
4-Hydroxy-3-methoxyphenylacetic acid	1.28	0.50

Determination of Radioactivity

Measurement of radioactivity was performed in a liquid scintillation counter using 5 g 2-(4'-tert.-butylphenyl)-5-(4''-biphenyl)-1,3,4-oxadiazole (butyl-PBD) per liter of toluene. Hydrophilic compounds were counted in a scintillation solution similar to that described by Bray [17]: 50 g naphthalene, 5 g butyl-PBD, 100 ml methanol and 900 ml dioxane. The radioactivity on paper chromatograms was determined in a chromatogram-scanner supplied with a Geiger-Müller detector tube (end-window type).

RESULTS

Biosynthesis of 2-Hydroxyphenylacetic Acid

The data in Table 1 summarize the results of feeding experiments in which ^{14}C-labelled compounds were administered to detached leaves of *A. chinensis*. The plants were 4—5 weeks old and started to bear flower buds. Not surprisingly, shikimic acid and certain phenylpropane derivatives showed the greatest efficiency of incorporation of ^{14}C into the 2-hydroxyphenylacetic acid. Phenylacetic acid and phenylpyruvic acid appeared to be the best precursors. Only

Table 1. *Administrations of various possible precursors of 2-hydroxyphenylacetic acid in* A. chinensis

Compound fed			2-Hydroxyphenylacetic acid		
	mg	μC	mg	μC	% ^{14}C converted
[U-^{14}C]Shikimic acid	5	10	11	0.5	5.0
DL-[β-^{14}C]Phenylalanine	5	25	15	2.9	11.6
[β-^{14}C]Cinnamic acid	8	25	8	< 0.02	< 0.01
[β-^{14}C]Phenylpyruvic acid	5	25	12	2.1	8.4
[β-^{14}C]Phenylpropionic acid	10	10	7	< 0.01	< 0.1
[*carboxyl*-^{14}C]Phenylacetic acid	3	25	16	3.4	13.6
[1-^{14}C]Acetic acid	2	25	9	0.2	0.8
[β-^{14}C]*o*-Coumaric acid	5	10	6	0.05	0.5
[β-^{14}C]Melilotic acid	6	10	7	< 0.01	< 0.1
D-[U-^{14}C]Glucose	2	10	9	0.3	3

a low incorporation of the tracer took place with labelled acetate; no incorporation of cinnamic acid or *o*-coumaric acid could be found. Likewise phenylpropionic acid and 2-hydroxyphenylpropionic acid (melilotic acid), known as metabolites of cinnamic acid in higher plants, were poor precursors. Similar results were also obtained with *Sinapis alba*.

Phenylacetic acid can be converted to 2-hydroxyphenylacetic acid by simple hydroxylation. Other possible pathways for its formation must also be taken into account. Thus, phenylpyruvic acid could be transformed to 2-hydroxyphenylacetic acid *via* 2-hydroxyphenylpyruvic acid or β-2-hydroxyphenylethyl amine and also a direct transformation from phenylpyruvic acid to 2-hydroxyphenylacetic acid could be considered. The percent incorporation of 2-hydroxyphenylacetic acid obtained from leaves fed with phenylpyruvic acid did not decrease when inactive 2-hydroxyphenylpyruvic acid was added to the solution to be infused.

If a direct conversion would be operative, it probably should involve a migration of the side chain analogous to the reaction catalyzed by phenylpyruvate oxidase from mammalian tissues [8]. Therefore, L-[4-^{3}H]phenylalanine was fed to the plants in order to label the original paraposition. In experiment 1, Table 2, the formation of [5-^{3}H]2-hydroxyphenylacetic acid from L-[4-^{3}H]phenylalanine fed to the leaves could be shown by the fact that the former compound loses all its activity on bromination. This finding is consistent with a migration of the side chain from the original position 1 of the ring to a vicinal position. Accordingly, a direct transformation of phenylpyruvic acid to 2-hydroxyphenylacetic acid appears to be the most likely pathway. No formation of radioactive phenylacetic acid was detectable when L-[^{14}C]phenylalanine was administered to the plants.

Experiment 2 revealed that a transformation *via* phenylacetic acid is not operative under physiological conditions. [4-^{3}H]Phenylacetic acid was fed to plants of *A. chinensis*. The 2-hydroxyphenylacetic acid isolated from these plants was labelled in position 4. Since here, in contrast to experiment 1 where phenylalanine was fed, no migration occurred, the phenylacetic acid pathway cannot play an important role.

Table 2. *Bromination of [4-^{3}H]- and [5-^{3}H]2-hydroxyphenylacetic acid*

In experiment 1 2-hydroxyphenylacetic acid was formed from L-[4-^{3}H]phenylalanine *in vivo*, in experiment 2 2-hydroxyphenylacetic acid was isolated after feeding of [4-^{3}H]-phenylacetic acid and in experiment 3 hydroxylation of [4-^{3}H]phenylacetic acid was carried out using the system peroxidase—dihydroxyfumarate—O_2. 2-Hydroxyphenylacetic acid thus obtained was diluted by inactive material, recrystallized and then brominated

Compound	Specific activity of the ^{3}H-labelled compound		
	expt. 1	expt. 2	expt. 3
	dis./min/μmole		
2-Hydroxyphenylacetic acid			
after 3rd crystallization	390	111	573
after 4th crystallization	385	106	583
3,5-Dibromo-2-hydroxyphenylacetic acid			
after 2nd crystallization	12	100	550
after 3rd crystallization	8	106	575

In experiment 3 nonspecific hydroxylation of [4-^{3}H]phenylacetic acid was carried out *in vitro*. The system plant peroxidase—dihydroxyfumarate—O_2 yielded mainly labelled 2-hydroxyphenylacetic acid. 3,5-Dibromo-2-hydroxyphenylacetic acid formed by bromination exhibited the same specific activity as the starting material, indicating that position 4 was labelled.

Occurrence and Biosynthesis of other Hydroxyphenylacetic Acids

During the reported work on the biosynthesis of 2-hydroxyphenylacetic acid, further phenolic compounds were detected which also derive from L-phenylalanine. Starting from a large amount of roots of *A. chinensis*, it was possible to separate one of these compounds from other phenols by preparative paper chromatography and fractional microsublimation. According to the analytical data (microanalysis, colour reactions and chromatographic behaviour)

this compound was assumed to be 2,3-dihydroxyphenylacetic acid. This was established by comparison with an authentic sample of this compound, synthesized from *o*-vanillin and hydantoin *via* 2-hydroxy-3-methoxy-benzalhydantoin, 2-hydroxy-3-methoxyphenylpyruvic acid and 2-hydroxy-3-methoxyphenylacetic acid (*cf.* Methods). A second compound could later be isolated from the same extract and proved to be 2-hydroxy-3-methoxy-phenylacetic acid as shown by comparison with a synthesized sample of this product.

The biosynthesis of these two compounds could easily be examined by feeding L-[β-^{14}C]phenylalanine and [U-^{14}C]2-hydroxyphenylacetic acid to *A. chinensis* and *S. alba*. Employing various periods of metabolism (Table 3) the following biosynthetic pathway could be established: phenylpyruvic acid → 2-hydroxyphenylacetic acid → 2,3-dihydroxyphenylacetic acid → 2-hydroxy-3-methoxyphenylacetic acid. In analogy to similar biosyntheses, it was imaginable that this pathway involves some of the named intermediates in glycosidic linkage. Utilizing the solubility in ether for differentiation between free and "bound" acids it could be shown that glycosides do not play an important role in these transformations.

In order to compare this route from phenylpyruvic acid with the biosynthesis and metabolism of 4-hydroxyphenylacetic acid, some possible precursors were fed to *A. chinensis*. Shikimic acid was converted into 4-hydroxyphenylacetic acid to 1%. 4-Hydroxyphenylpyruvic acid is another precursor which is incorporated into 4-hydroxyphenylacetic acid three times better than shikimic acid. The finding that no measurable amount of labelled 4-hydroxyphenylacetic acid was formed from [*carboxyl*-^{14}C]-phenylacetic acid, DL-[β-^{14}C]phenylalanine and (β-^{14}C)phenylpyruvic acid demonstrates that shikimic acid and 4-hydroxyphenylpyruvic acid are the only natural precursors of 4-hydroxyphenylacetic acid in the investigated plants.

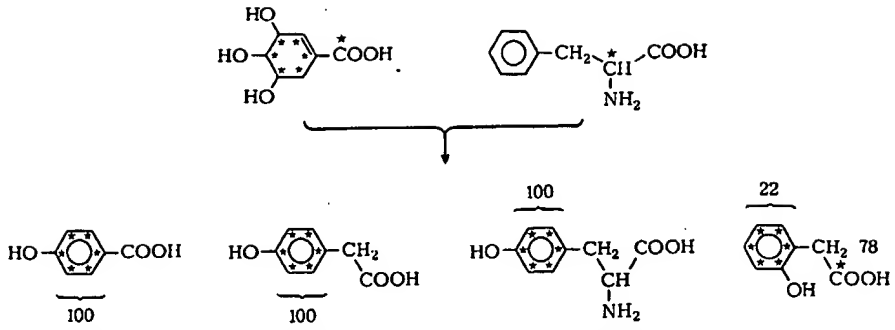
Feeding of DL-[α-^{14}C]tyrosine yielded labelled 4-hydroxyphenylacetic acid (7.5% incorporation) and two further radioactive spots which were identified as 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid. More accurate information was provided by paper chromatographic analysis of extracts of *A. chinensis* showing that this plant material contains 2-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid in a ratio of 15:1. [U-^{14}C]4-Hydroxyphenylacetic acid infused into the plant leaves was converted to [^{14}C]-3,4-dihydroxyphenylacetic acid with 15% incorporation.

Comparison of the Biosynthetic Routes

For the comparison of several biosynthetic routes a competition experiment involving the simultaneous feeding of [U-^{14}C]shikimic acid and DL-[α-^{14}C]phenylalanine was made. After 24 hours 4-hydroxybenzoic acid, L-tyrosine, 4-hydroxyphenylacetic acid, and 2-hydroxyphenylacetic acid could be isolated (Scheme 1). A selective degradation of these compounds revealed that in 4-hydroxybenzoic acid only the aromatic ring was labelled and less than 1% of the entire activity of 4-hydroxybenzoic acid was localized in the carboxyl group. 4-Hydroxyphenylacetic acid contained almost all the activity in the ring; less than 1% was found in the carboxyl group and in the methylene group. The 2-hydroxyphenylacetic acid obtained, however, was labelled in the carboxyl group (78%) and in the ring (22%). No radioactivity could be detected in the methylene group (α-position). The results mentioned regarding 4-hydroxybenzoic acid show that this acid derives from phenylpropane units and rule out a direct transformation of shikimic acid or its derivatives into 4-hydroxybenzoic acid, because otherwise the carboxyl group of shikimic acid would be retained. The further results prove that 4-hydroxyphenylacetic acid is exclusively formed from shikimic acid and not from L-phenylalanine *via* L-tyrosine and also show that 2-hydroxyphenylacetic acid is predominantly a metabolite of L-phenylalanine.

Table 3. *Feeding experiments in which* L-*[β-^{14}C]phenylalanine and [U-^{14}C]2-hydroxyphenylacetic acid were administered to leaves (10 g fresh weight) of* A. chinensis *(A) and* S. alba *(S)*

	Compound fed	Amount		Time	Activity found in the isolated compound		
					2-Hydroxy-phenylacetic acid	2,3-Dihydroxy-phenylacetic acid	2-Hydroxy-3-methoxyphenyl-acetic acid
		mg	μC	h	%	%	%
A	L-[β-^{14}C]Phenylalanine	1.5	4.0	5	1.2	0.1	0.01
A	L-[β-^{14}C]Phenylalanine	2.5	6.7	35	15.7	4.0	0.8
S	L-[β-^{14}C]Phenylalanine	1.5	4.0	50	1.5	1.1	0.2
A	[U-^{14}C]2-Hydroxyphenylacetic acid	3.0	5.1	5	71.0	21.8	0.1
A	[U-^{14}C]2-Hydroxyphenylacetic acid	3.0	5.1	35	39.1	45.9	3.8
S	[U-^{14}C]2-Hydroxyphenylacetic acid	3.0	5.1	35	32.0	41.1	6.8



Scheme 1. *Competition experiments in* A. chinensis. After simultaneous feeding of [U-^{14}C]shikimic acid and DL-[α-^{14}C]phenylalanine the plants were allowed to metabolize these compounds during a period of 15 hours. 4-Hydroxybenzoic acid, 4-hydroxyphenylacetic acid, L-tyrosine, and 2-hydroxyphenylacetic acid were isolated, diluted, and chemically degraded as described. The activity found in the various C-atoms is specified in percentage of the entire labelling of the compound formed

Nonspecific Hydroxylation of Phenylacetic Acid in vitro

These experiments were performed with the known model hydroxylating system peroxidase—endiol—O_2. As endiol, dihydroxyfumarate has been utilized as well as pentahydroxycyclohexanones. During a period of 30 min, at pH 8.0 and 20°, no great differences in the hydroxylation patterns could be observed, whether dihydroxyfumarate or 2,4,6/3,5-pentahydroxycyclohexanone had been used. 2-Hydroxyphenylacetic acid was the main product (1—5 % radioactive yield) besides small amounts of the meta- and para-isomer, 2,3-dihydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid. When [*carboxyl*-^{14}C]phenylacetic acid with high specific activity was used for these experiments at least five further radioactive peaks could be detected on the paper chromatogram.

Further Metabolism of Hydroxyphenylacetic Acids

Another series of feeding experiments were performed in order to demonstrate that hydroxyphenylacetic acids cannot be considered as metabolic end products of higher plants but are subjected to further metabolism. [U-^{14}C]2-Hydroxyphenylacetic acid, formed from [U-^{14}C]phenylacetic acid with peroxidase—endiol—O_2 and purified by paper chromatography, was infused into plants of *A. chinensis* and *S. alba* and the excretion of $^{14}CO_2$ was measured. Finally, the plant material was worked up and the radioactivity of D-glucose was determined. While the excretion of $^{14}CO_2$ was less than 1 % of the radioactivity fed, 5 % of the radioactivity were localized in D-glucose after a period of 5 days.

Furthermore, all three dihydroxyphenylacetic acids identified in *A. chinensis*, 2,3-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 2,5-dihydroxyphenylacetic acid, were fed in labelled form (obtained biosynthetically) into plants of *S. alba* and *A. chinensis*. In all cases radioactive D-glucose was detectable. These findings are consistent with the hypothesis that all three aromatic acids can be degraded to aliphatic acids by ring cleavage.

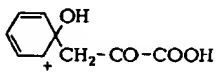
DISCUSSION

Various species of *Aspergillus* [18] and *Pseudomonas* [19] convert phenylacetic acid primarily to 4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid. Cell-free extracts from *Penicillium chrysogenum* treated with phenylacetic acid form 4-, and 2-hydroxyphenylacetic acid [20]. According to the results presented here some higher plants, however, seem to oxidize phenylacetic acid almost exclusively to 2-hydroxyphenylacetic acid and to 2,3-dihydroxyphenylacetic acid. Whether this is a general pathway of phenylacetic acid in higher plants remains open to further studies. So far, little is known on the occurrence and biosynthesis of phenylacetic acid in plants. It is found as ester in essential oils, and Moore, Subba Rao and Towers [21] describe its formation from phenylalanine in *Schizophyllum commune*. Since phenylacetic acid is immediately oxidized to 2-hydroxyphenylacetic acid in the plants examined here, and 2-hydroxyphenylacetic acid is formed from phenylpyruvic acid only by a mechanism involving migration of the side chain, it can be concluded that no formation of phenylacetic acid had taken place. Accordingly, no radioactive phenylacetic acid was detectable when labelled phenylpyruvic acid was fed. Furthermore due to the lack of phenylacetic acid, 4-hydroxyphenylacetic acid found in many higher plants derives most likely from 4-hydroxyphenylpyruvic acid only and not from phenylacetic acid.

Experiments with hydroxylating model systems performed in other laboratories demonstrated the formation of 2-, 3-, and 4-hydroxyphenylacetic acids in various ratios [7, 20]. No phenolic compounds were produced from phenylacetic acid by liver microsomal systems [7]. The experiments with peroxidase re-

ported here go a step farther as they also include the formation of the dihydroxy compounds and give a broader outlook on the hydroxylation patterns of phenylacetic acid.

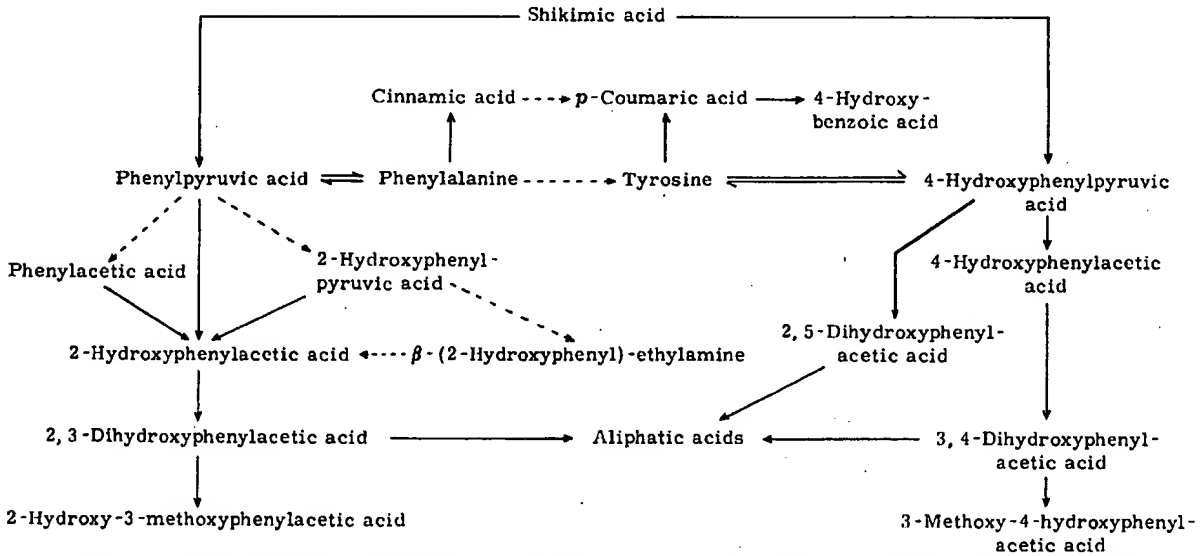
The conversion of L-[4-^{3}H]phenylalanine to [5-^{3}H]2-hydroxyphenylacetic acid taking place in *Astilbe* and presumably in many other higher plants is compatible with the findings of Taniguchi, Kappe and Armstrong [22] who reported a transformation of 4-fluorophenylpyruvate to 2-hydroxy-5-fluorophenylacetic acid by a mammalian enzyme. This enzyme seems to act as phenylpyruvic acid oxidase. The conversion takes place without the formation of any detectable intermediates. In our experiments with *A. chinensis* also, no 2-hydroxyphenylpyruvic acid could be found suggesting that the conversions in mammalian liver and in higher plants both proceed by the same way. The reaction sequence *via* 2-hydroxyphenylalanine or the corresponding amine seems to function in animals only. A detailed mechanism was proposed for the conversion of 4-hydroxyphenylpyruvic acid to homogentisic acid [23]. Recent studies on the hydroxylation of aromatic compounds [24,25] strongly indicate the formation of a cationoid intermediate; thus an enzyme bound intermediate may be formulated:



which may undergo hydroxylation induced migration followed by oxidative decarboxylation of the side chain. But at the moment it is probably premature to postulate a mechanism.

The biosynthetic pathways leading to the different hydroxyphenylacetic acids (Scheme 2) reveal some analogy to the pattern of the biological hydroxylation of benzoic acids [26] and phenylpropionic acids [27]. 2-Hydroxyphenylalkanoic acids are converted to 2,3-dihydroxyphenylalkanoic acids and 2-hydroxy-3-methoxy-phenylalkanoic acids, whereas the oxidation of 4-hydroxyphenylacetic acid to 3,4-dihydroxyphenylacetic acid and the methylation to 3-methoxy-4-hydroxyphenylacetic acid has no equivalent in the metabolism of benzoic acid, at least not as a main route in higher plants.

The degradation of L-phenylalanine in animals is known to proceed *via* L-tyrosine and homogentisic acid [28]. L-Phenylalanine hydroxylase has been found in higher plants, too [12]. In *Astilbe*, however, L-phenylalanine is almost exclusively converted to 2-hydroxyphenylacetic acid and 2,3-dihydroxyphenylacetic acid, but not to 4-hydroxyphenylacetic acid. Therefore the hydroxylation of L-phenylalanine to L-tyrosine cannot play an important role, or may not function in this plant, as it was found in patients with phenylketonuria. Thus, in *Astilbe* the lack of L-phenylalanine hydroxylase may be the reason for the increased pool of L-phenylalanine and the considerable formation of 2-hydroxyphenylacetic acid. This idea is supported by the findings that [^{14}C]4-hydroxybenzoic acid is derived from L-[U-^{14}C]phenylalanine *via* cinnamic acid and *p*-coumaric acid only, whereas no formation of L-[^{14}C]tyrosine was found. In agreement to that, leaves of *A. chinensis* exhibit high activity of L-phenylalanine ammonia lyase, but in contrast to some other Saxifragaceae investigated, no L-phenylalanine hydroxylase activity could be detected. In the course of taxonomical studies [4] 2-hydroxyphenylacetic acid had been found to be diagnostic of the genus *Astilbe*. Its occurrence may be caused by the enzymatic regulation of the pool



Scheme 2. *Possible pathways and established routes leading to the formation of hydroxyphenylacetic acids in* A. chinensis

size of L-phenylalanine, or can be regarded as a consequence of an "inborn error of metabolism". Since in other plants (*S. alba, Chrysanthemum leucanthemum)*, phenylpyruvic acid is converted to 2-hydroxyphenylacetic acid too, probably also involving a migration of the side chain, it must be assumed that many higher plants possess the enzymatic equipment to form 2-hydroxyphenylacetic acid, although the compound is normally not detectable in these species. Previous analyses of species of the family Saxifragaceae showed that in contrast to other genera of this family, the genus *Astilbe* contains very low concentration of *p*-coumaric acid and ferulic acid [4]. It is very tempting to suggest that this could be due to the lack of L-phenylalanine hydroxylase.

All the evidence which is so for available supports the concept that the regulation of the biosynthesis of 2-hydroxyphenylacetic acid and 4-hydroxyphenylacetic acid and its metabolites cannot occur on the level of phenylpropane derivatives or phenylacetic acid, but takes place earlier. Most likely the regulation of the pathway leading to 2-hydroxyphenylacetic acid or to 4-hydroxyphenylacetic acid is closely associated with two allosteric proteins, "P-protein" and "T-protein" [29]. "P-protein" contains chorismate mutase P and prephenate dehydratase whereas "T-protein" acts as chorismate mutase T and prephenate dehydrogenase. Both, "P-protein" and "T-protein", are found to be sensitive to end-product inhibition by tyrosine and phenylalanine [29]; in earlier studies with prephenate dehydrogenase alone no inhibition by tyrosine could be found [30].

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REFERENCES

1. Boscott, R. J., and Bickel, H., *Scand. J. Clin. Lab. Invest.* 5 (1953) 380.
2. King, N. K., and Hambly, A. N., *Roy. Australian Chem. Inst. J. Proc.* 17 (1950) 403.
3. Kindl, H., and Billek, G., *Nature*, 194 (1962) 579.
4. Billek, G., and Kindl, H., *Monatsh. Chem.* 93 (1962) 85.
5. Udenfried, S., and Mitoma, C., in *Symposium on Amino Acid Metabolism* (edited by W. D. McElroy and H. B. Glass), Johns Hopkins Press, Baltimore 1955, p. 876.
6. Tashian, R. E., *Science*, 129 (1959) 1553.
7. Armstrong, M. D., and Shaw, K. N. F., *J. Biol. Chem.* 213 (1955) 805.
8. Taniguchi, K., and Armstrong, M. D., *J. Biol. Chem.* 238 (1963) 4091.
9. Billek, G., and Kindl, H., *Atompraxis*, 8 (1962) 167.
10. Murray, A., and Williams, D. L., *Organic Synthesis with Isotopes*, Interscience Publishers, New York 1956.
11. Billek, G., Kindl, H., Schimpl, A., and Schmook, F. P., *J. Labelled Compds.*, in press.
12. Nair, P. M., and Vining, L. C., *Phytochemistry*, 4 (1965) 401.
13. Koukol, J., and Conn, E. E., *J. Biol. Chem.* 236 (1961) 2692.
14. Kindl, H., and Hoffmann-Ostenhof, O., *Phytochemistry*, 5 (1966) 1091.
15. Billek, G., *Monatsh. Chem.* 92 (1961) 343.
16. Kindl, H., *Monatsh. Chem.* 95 (1964) 439.
17. Bray, G. A., *Anal. Biochem.* 1 (1960) 279.
18. Kluyver, A. J., van Zijp, J. C. M., and van Leeuwenhoeck, A., *J. Mikrobiol. Serol.* 17 (1951) 315.
19. Blakeley, E. R., Kurz, W., Halvorson, H., and Simpson, F. J., *Can. J. Microbiol.* 13 (1967) 147.
20. Nyiri, L., Erdelyi, A., and Lengyel, Z. L., *Z. Allg. Mikrobiol.* 6 (1966) 379.
21. Moore, K., Subba Rao, P. V., and Towers, G. H. N., *Life Sci.* 6 (1967) 2692.
22. Taniguchi, K., Kappe, T., and Armstrong, M. D., *J. Biol. Chem.* 239 (1964) 3389.
23. Daly, J. W., and Witkop, B., *Angew. Chem., Intern. Ed.* 2 (1963) 421.
24. Ullrich, V., Wolf, J., Amadori, E., and Staudinger, Hj., *Z. Physiol. Chem.* 349 (1968) 85.
25. Guroff, G., Dalay, J. W., Jerina, D. M., Renson, J., Witkop, B., and Udenfried, S., *Science*, 157 (1967) 1524.
26. Kindl, H., and Billek, G., *Österr. Chem. Zeitg.* 63 (1962) 290.
27. Dagley, S., Chapman, P. J., and Gibson, D. T., *Biochem. J.* 97 (1965) 643.
28. Meister, A., *Biochemistry of the Amino Acids*, Academic Press, New York 1965, Vol. II, p. 884.
29. Cotton, R. G. H., and Gibson, F., *Biochim. Biophys. Acta*, 156 (1968) 187.
30. Gamborg, O. L., and Keeley, F. W., *Biochim. Biophys. Acta*, 115 (1966) 65.

H. Kindl
Lehrkanzel für Biochemie der Universität
Währingerstraße 38, A-1090 Wien, Austria